Polyanions Decelerate the Kinetics of Positively Charged Gramicidin Channels as Shown by Sensitized Photoinactivation

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ABSTRACT The effects of different anionic polymers on the kinetic properties of ionic channels formed by neutral gramicidin A (gA) and its positively charged analogs gramicidin-*tris*(2-aminoethyl)amine (gram-TAEA) and gramicidin-ethylenediamine (gram-EDA) in a bilayer lipid membrane were studied using a method of sensitized photoinactivation. The addition of Konig's polyanion caused substantial deceleration of the photoinactivation kinetics of gram-TAEA channels, which expose three positive charges to the aqueous phase at both sides of the membrane. In contrast, channels formed of gram-EDA, which exposes one positive charge, and neutral gA channels were insensitive to Konig's polyanion. The effect strongly depended on the nature of the polyanion added, namely: DNA, RNA, polyacrylic acid, and polyglutamic acid were inactive, whereas modified polyacrylic acid induced deceleration of the channel kinetics at high concentrations. In addition, DNA was able to prevent the action of Konig's polyanion. In single-channel experiments, the addition of Konig's polyanion resulted in the appearance of long-lived gram-TAEA channels. The deceleration of the gram-TAEA channel kinetics was ascribed to electrostatic interaction of the polyanion with gram-TAEA that reduces the mobility of gram-TAEA monomers and dimers in the membrane via clustering of channels.

INTRODUCTION

Studies of how polyelectrolytes interact with membranes are important for understanding and improving the process of gene delivery. Since the demonstration that cationic liposomes can enhance transfection (Felgner et al., 1987), substantial efforts have gone into characterizing interaction between DNA and cationic membranes (Kinnunen et al., 1993; Koiv et al., 1994; Mitrakos and Macdonald, 1996, 1998; Hirsch-Lerner and Barenholz, 1998; Meidan et al., 2000; Subramanian et al., 2000; Safinya, 2001).

Membrane ion channels can act as exquisitely sensitive sensor elements that report on polyelectrolyte/membrane interactions. There is a substantial body of evidence demonstrating modulation of the activity of certain pore-forming proteins by polyanions, e.g., by DNA sequences (Wright and Harding, 2000; Tosteson et al., 2001), and by Konig's polyanion (Konig et al., 1982; Colombini et al., 1987; Benz et al., 1988; Tedeschi et al., 1987; Tedeschi and Kinnally, 1987; Mannella and Guo, 1990; Mirzabekov et al., 1993). Transport of polyelectrolytes, in particular of nucleic acid fragments, through proteinaceous pores in membranes has also been reported (Kasianowicz et al., 1996; Szabo et al., 1997; Hanss et al., 1998; Akeson et al., 1999; Meller et al., 2000, 2001; Henrickson et al., 2000; Vercoutere et al., 2001).

The gramicidin channel is a structurally well-defined and functionally well-characterized system (Woolley and Wallace, 1992; Busath, 1993; Koeppe and Andersen, 1996; Andersen et al., 1999) that can be engineered to act as a sensor for a variety of purposes, e.g., pH measurement (Borisenko et al., 2002) and avidin/streptavidin detection (Cornell et al., 1997, 1999; Suarez et al., 1998; Rokitskaya et al., 2000a; Futaki et al., 2001). Previously, we used O-pyromellitylgramicidin (OPg), a gramicidin derivative with three negative charges at the C-terminal end, to investigate the interaction of polycations with membranes (Krylov et al., 1998, 2000).

Because the interaction of polyanions with membranes is expected to be influenced by the concentration and charge density of cationic species in the membrane (Safinya, 2001), we prepared gramicidin analogs bearing one (gramicidin-EDA) and three (gramicidin-TAEA) positive charges and studied the effect of various synthetic and natural polyanions on the behavior of the channels formed by the gramicidin analogs in planar bilayer lipid membranes using the methods of sensitized photoinactivation and single-channel recording.

MATERIALS AND METHODS

Peptide synthesis

Gramicidin-ethylenediamine (gram-EDA, Fig. 1) was prepared as described previously (Jaikaran et al., 1995). Gramicidin-*tris*(2-aminoethyl) amine (gram-TAEA, Fig. 1) was synthesized in a similar fashion. Commercial gramicidin D (20 mmol) was esterified (1 h, 4°C) with *p*-nitrophenyl chloroformate (200 mmol) in dry tetrahydrofuran (2 ml) containing triethylamine (TEA) (100 ml). Then *tris*(2-aminoethyl) amine in a 150-fold molar excess was added, and the reaction mixture was stirred for 20 min at room temperature. The solution then was filtered, then dried on a rotaevaporator. The product was then separated by gel-filtration using LH-20 in methanol and by ion exchange chromatography with an AG-MP 50 column (Weiss and Koeppe, 1985). Finally, a thin-layer chromatography separation (chloroform/methanol/water, 65:25:4) was applied to give gram-TAEA ($0 < R_f < 0.29$), which was characterized by ultraviolet and mass

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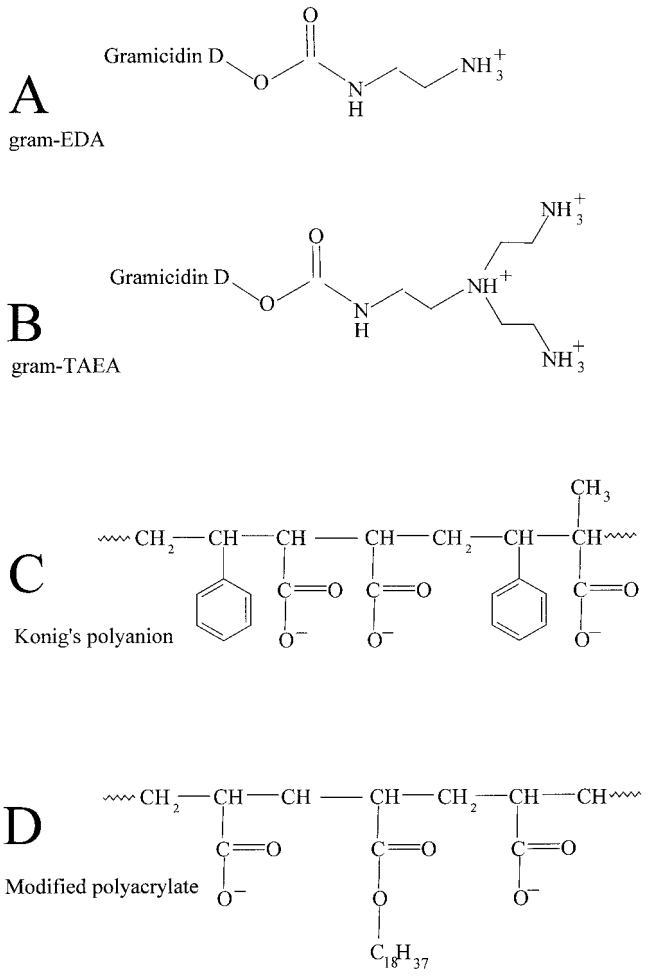

FIGURE 1 Chemical structure of (*A*) gramicidin-ethylenediamine (gram-EDA), (*B*) gramicidin-*tris*(2-aminoethyl)amine (gram-TAEA), (*C*) a repeat unit of Konig's polyanion, and (*D*) a repeat unit of modified polyacrylic acid.

spectrometry. MS (maldi positive): the major peak was 2054, corresponding to gramA-TAEA. Other peaks, corresponding to gramB-TAEA and gramC-TAEA, were of significantly lower intensity.

Polyanions synthesis

Konig's polyanion (the copolymer of styrene, maleic acid, and methacrylic acid, Fig. 1) was synthesized according to Konig et al. (1977). The monomeric composition of the final product was estimated using infrared and ultraviolet spectroscopy, and the molecular weight of the polymer was evaluated using gel-permeation chromatography in ethanol with Waters polyethylene oxide-markers as molecular weight standards.

The copolymer of acrylic acid with stearylacrylate (the modified polyacrylic acid, Fig. 1) was synthesized by esterification of polyacrylic acid by octadecyl alcohol in dioxane in the presence of dicyclohexylcarbodiimide. Polyacrylic acid, 100 mg, (MW 6700; 1.39 mmol of acrylic units) were dissolved in 5 ml dry dioxane at 80°C and 300 mg of octadecyl alcohol (1.11 mmol) (Sigma, St. Louis, MO) and 70 mg dicyclohexyl carbodiimide (0.35 mmol) (Aldrich, Milwaukee, WI) were added. The mixture was incubated at 60°C for 5 days. The solution was cooled and precipitated octadecyl alcohol was separated by filtration. The filtrate was collected and mixed with 4 ml 0.1 M KOH solution in methanol to obtain potassium polyacrylate, which is insoluble in organic solvents. The mixture was then mixed with hexane, and polyacrylate was allowed to precipitate overnight at room temperature. The polymer was collected by centrifugation, washed with hexane, and dried in vacuo. Then the polymer was dissolved in 0.1 M borate buffer, pH 8.5, and the solution was centrifuged to separate the polymer from admixtures of water-insoluble octadecyl alcohol. The solution was then acidified with HCl to pH 2.5–3.0 and dialyzed extensively against distilled water to obtain the acidic form of the polymer, which is practically insoluble in water. The final product was lyophilized and the amount of the attached ester groups was estimated using alkaline titration. For this purpose, 10 mg of the polymer was dissolved in 2 ml 0.09 M NaOH and the content of carboxylic groups was estimated using a pH-stat (Radiometer, Copenhagen, Denmark) titration. The apparent degree of modification was ~18%, i.e., each polymer chain contained ~16 octadecyl radicals.

Single-channel measurements

Peptides (~10 nM in methanol) were added to membranes formed from diphytanoyl-phospatidylcholine/decane (50 mg/ml). Lipid bilayers were formed across an ~100-μm hole in a polypropylene pipet tip by painting a solution of lipid in decane. The pipet tip was mounted in a Teflon cell through a small hole in the back face. The front face of this cell had a removable circular glass window. Silver/silver chloride electrodes were placed in the pipet tip and a cylindrical well drilled from the top of the cell. Symmetrical buffered (0.5 mM BES, pH 7) KCl (0.1 M) solutions with and without Konig's polyanion were used. All measurements were made at room temperature.

The current through lipid bilayers containing the gramicidin derivative was measured, and the voltage was set using an Axopatch 1D patch-clamp amplifier (Axon Instruments, Union City, CA) controlled by Synapse (Synergistic Research Systems) software. Single-channel events were recorded for a period of several hours for each set of experimental conditions. Data were filtered at 100 Hz, sampled at 1 kHz, stored directly to disk, and analyzed using Synapse and Igor software (Wavemetrics, Inc., Lake Oswego, OR). The mean lifetimes and current amplitudes were determined by fitting appropriate functions to corresponding histograms using the program Mac-Tac (Version 2.0, Instrutech Corp., Port Washington, N.Y.).

Photoinactivation measurements

Bilayer lipid membranes (BLMs) were formed from a 2% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) by the brush technique (Mueller et al., 1963) on a 0.8-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 50 mM (unless otherwise stated) KCl (Fluka, Buchs, Switzerland), 10 mM MES (Sigma), 10 mM TRIS (Sigma) and 0.1 mM EDTA at pH 7.0. Gram-TAEA, gram-EDA, or gramicidin A (Sigma) (Cifu et al., 1992) was added from stock solutions in ethanol to the bathing solutions at both sides of the BLM and routinely incubated for 15 min with constant stirring. Polyanions were added to both compartments of the cell unless otherwise stated. Polyacrylic acids (MW 5000 and 250,000) were from Aldrich, poly-L-glutamic acid (MW 15,000) from Serva (Heidelberg, Germany). Calf thymus DNA (single- and double-stranded) and DNA from salmon testes were from Sigma. RNA from tobacco mosaic virus was a generous gift of Prof. D. B. Zorov, Moscow State University. Poly-L-lysine hydrobromide (MW 7500) was from Sigma. Experiments were carried out at room temperature (20–22°C). Aluminum trisulfophthalocyanine ($AlPcS_3$) was from Porphyrin Products (Logan, UT). $AlPcS_3$ was added to the bathing solution at the *trans*-side (the *cis*-side is the front side with respect to the flash lamp). The electric current (I) was recorded under voltage-clamp conditions. The currents were measured by means of a U5-11 amplifier (Moscow, Russia), digitized by using a LabPC 1200

(National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). Ag-AgCl electrodes were placed directly into the cell and a voltage of 30 mV (unless otherwise stated) was applied to the BLM. The value of the current was usually ~1 μA, which corresponded to 3×10^6 conducting channels in the bilayer. BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of ~400 mJ/cm^2 and flash duration < 2 ms. A glass filter cutting off light with wavelengths < 500 nm was placed in front of the flash lamp. To avoid electrical artifacts, the electrodes were covered by black plastic tubes.

In the presence of a photosensitizer (e.g., aluminum phthalocyanine or Rose Bengal, the dyes that sensitize singlet oxygen formation with high quantum yield), irradiation of BLM with visible light is known to decrease the gramicidin-mediated transmembrane current, I (Strassle and Stark, 1992; Rokitskaya et al., 1993). The decrease in the current is believed to result from damage to tryptophan residues of gramicidin (tryptophan oxidation or peptide fragmentation) (Strassle and Stark, 1992; Kunz et al., 1995) caused by reactive oxygen species that are generated upon excitation of a photosensitizer (Rokitskaya et al., 1996, 2000b). If BLM is illuminated with a single flash of visible light, I is a monoexponential function of time (Rokitskaya et al., 1996): $I(t) = (I_0 - I_\infty)\exp(-t/\tau) + I_\infty$, where I_0, I_∞, and τ are the initial current before illumination, the stationary level of the current established as a result of relaxation after the flash, and the characteristic time of photoinactivation, respectively. The relative amplitude of photoinactivation, α, is defined as $\alpha = (I_0 - I_\infty)/I_0$. Because the light-induced decrease in the gramicidin-mediated current is due to the reduction of the number of open channels (Rokitskaya et al., 1993; Kunz et al., 1995), α is equal to the damaged part of gramicidin channels. It should be noted that, in the presence of polyanions, the flash-induced decrease in the current was recorded 10–15 min after the addition of polyanions, when a new steady-state level of the current was reached.

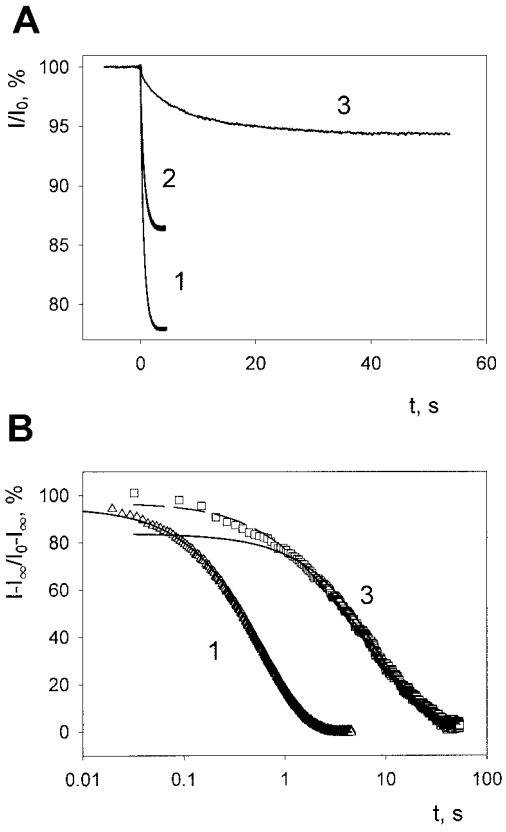

FIGURE 2 The kinetics of the decrease in the gram-TAEA-mediated current (I) through BLM in the presence of 1 μM $AlPcS_3$ (at the *trans* side) after a flash of visible light (at zero time) in the control (*curves 1* in panels *A* and *B*), and in the presence of 0.06 μg/ml Konig's polyanion at the *cis* side of BLM (*curve 2* of panel *A*), and at both sides of BLM (*curves 3* of panels *A* and *B*). The experimental data points were plotted as (I/I_0) 100% in panel *A* and replotted as $(I - I_\infty)100\%/(I_0 - I_\infty)$ versus the time (logarithmic scale) in panel *B*, where I_0 is the initial current and I_∞ is the stationary value of the current after a flash. Data were fitted with single exponentials (*solid curves*) with characteristic times $\tau = 0.60$ s (*curve 1*), $\tau = 0.67$ s (*curve 2*), and $\tau = 8.5$ s (*curve 3*). In the case of curve *3*, double-exponential fitting $I = I_0 + \alpha_1\exp(-t/\tau_1) + \alpha_2\exp(-t/\tau_2)$ (*panel B*, *dashed curve*) was also calculated with the following parameters: $\alpha_1 = 70\%$, $\tau_1 = 10.8$ s, $\alpha_2 = 30\%$, $\tau_2 = 1.5$ s. The initial value of the current (I_0) was ~1 μA.

RESULTS

To study channel kinetics of gram-TAEA, one of the positively charged analogs of gramicidin A, we applied a method of sensitized photoinactivation previously developed by Rokitskaya et al. (1996). It is shown in Fig. 2 that a visible light flash induced an irreversible decrease in the gram-TAEA-mediated current across BLM, if a photosensitizer (aluminum trisulfophthalocyanine) was added to the bathing solution. The time course of the current decrease (below called the kinetics of photoinactivation) was fitted well by a monoexponential curve similarly to the data obtained earlier for gramicidin A, with the characteristic time of photoinactivation (τ, the exponential factor of the curve) being close to that of gA. The addition of Konig's polyanion at one side of the BLM slightly increased the characteristic time of photoinactivation (from 0.60 to 0.67 s), and the result was independent of the side of the polyanion addition with respect to the photosensitizer side (*trans*). In contrast, the addition of the polyanion at both sides of the BLM produced a marked increase in τ up to 8.5 s (Fig. 2, *curve 3*). Figure 2 illustrates the data of typical measurements performed with a single membrane. In the presence of Konig's polyanion (*curve 3*) the kinetics of the current decrease displayed pronounced deviations from a monoexponential curve not seen in the control kinetics (*curve 1*). Subsequent addition of 0.1 mM poly-L-lysine to the bathing solutions completely reversed the polyanion effect on τ ($\tau = 0.61$ s, data not shown). In contrast to the photoinactivation kinetics of gram-TAEA possessing three positive charges, that of neutral gA was completely insensitive to additions of Konig's polyanion (data not shown).

It is seen from Fig. 2 *A* that Konig's polyanion not only slowed down the kinetics of gram-TAEA photoinactivation, but also decreased its relative amplitude. The reduction of the photoinactivation amplitude can be accounted for by quenching of reactive oxygen species by the polyanion. As has been shown by Rokitskaya et al.(1996), the characteristic time and the amplitude of photoinactivation represent two independent parameters; for instance, variation of the photosensitizer concentration alters markedly the amplitude

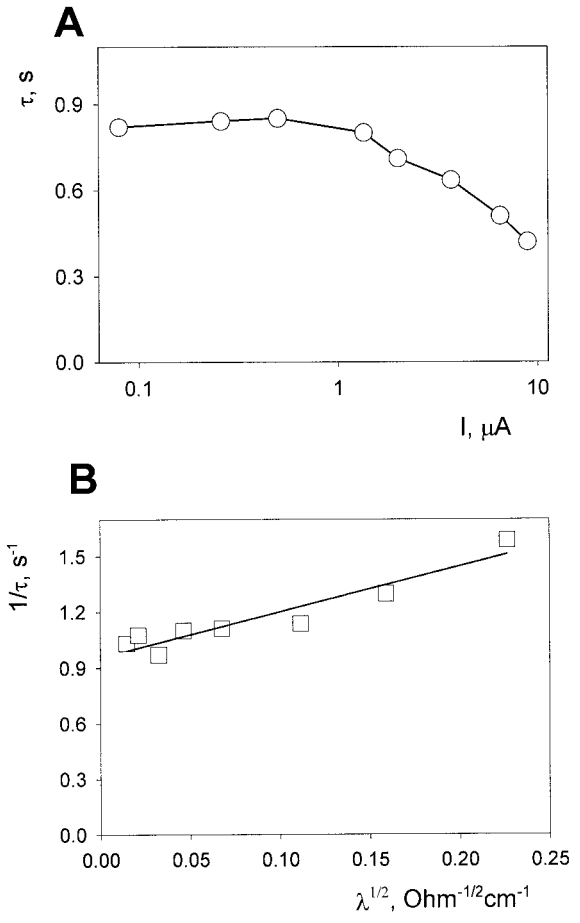

FIGURE 3 (*A*) The dependence of the characteristic time of gram-TAEA photoinactivation (τ) measured in the absence of Konig's polyanion on the BLM current at 50 mV. The current was varied by increasing the concentration of Gram-TAEA in the bathing solution. (*B*) The dependence of $1/\tau$ versus square root of the BLM conductance, which was used in calculation of the kinetic constants of Gram-TAEA. The buffer solution contained 100 mM KCl.

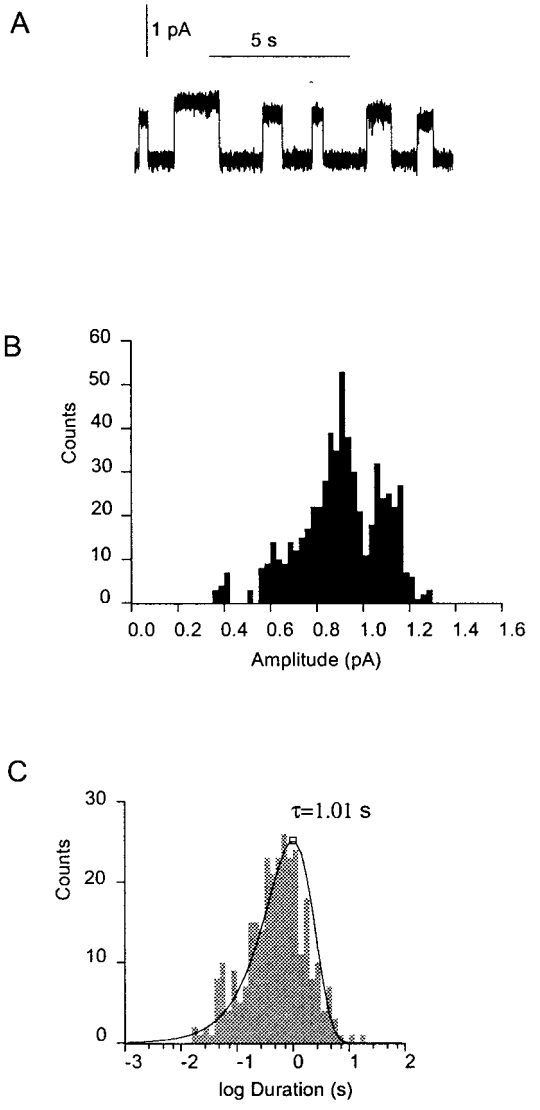

FIGURE 4 (*A*) Current trace, (*B*) single-channel current amplitude histogram, and (*C*) duration histogram of gram-TAEA. The currents were obtained at +100 mV. Several hundred single channels were characterized.

of photoinactivation, but does not change its characteristic time. The present study deals with the effect of polyanions on the characteristic time of photoinactivation, τ.

As described in the Appendix, the dependence of τ on the concentration of the channel former, plotted as $1/\tau$ versus square root of the BLM conductance (λ_∞), can be used to calculate the rate constants of the channel formation (K_R) and termination (K_D). Figure 3 presents this dependence for gram-TAEA. Using the data of Fig. 3 and Eq.10 of the Appendix, we obtain $K_R = (3.6 \pm 1.5) * 10^{12}$ mol^{-1}s^{-1}cm^2 and $K_D = (0.9 \pm 0.1)$ s^{-1} ($T = 23$°C).

The value of the gram-TAEA single-channel conductance (Λ) needed for the estimations of the rate constants was taken from single-channel recordings of gram-TAEA shown in Fig. 4. The predominant value of the conductance (Λ) was determined to be 10 pS (0.1 M KCl, DPhPC) and the channel lifetime was 1.0 s. The single-channel current-amplitude histogram indicated the existence at least of two open sublevels, as was observed for other gramicidin analogs with modified C-terminus (Woolley et al., 1995). The value of the single-channel lifetime of gram-TAEA is close to the ($1/K_D$) value calculated from the photoinactivation data, thus demonstrating the compatibility of the two methods. The addition of 100 ng/ml Konig's polyanion did not significantly alter the average single-channel conductance as seen from the corresponding amplitude histogram (Fig. 5). However, this addition led to the appearance of a subset of channel events of much longer duration (see Fig. 5 *A*, *recording 2*). These events manifested themselves in the time histogram as a small shoulder at long time durations (Fig. 5 *C*). These channel events also exhibited fast flickering (Fig. 5 *A*).

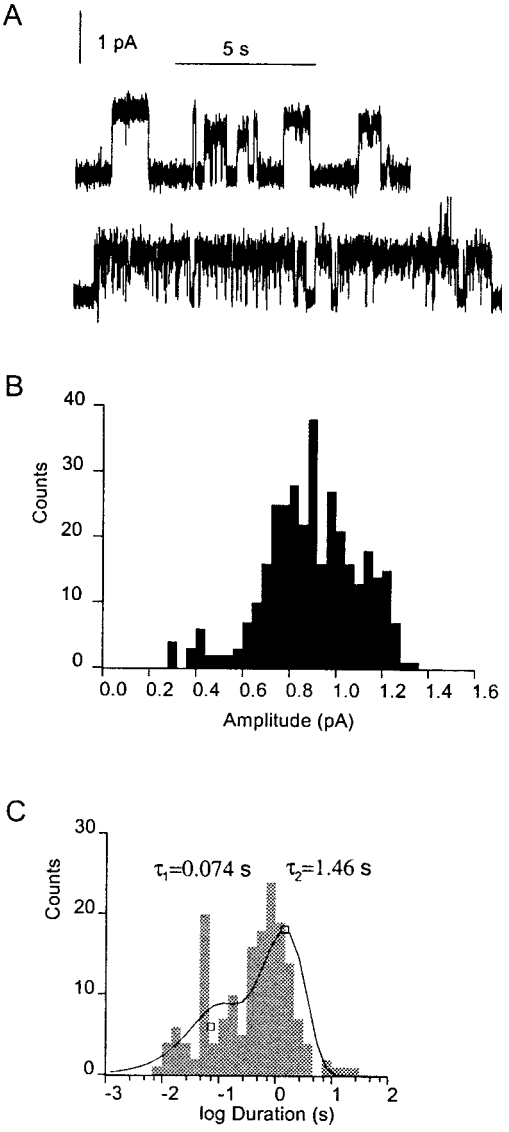

FIGURE 5 (*A*) Current traces, (*B*) single-channel current amplitude histogram, and (*C*) duration histogram of gram-TAEA in the presence of 100 ng/ml of Konig's polyanion. The two rows of current traces show two representative parts of the single-channel recording. Several hundred single channels were characterized.

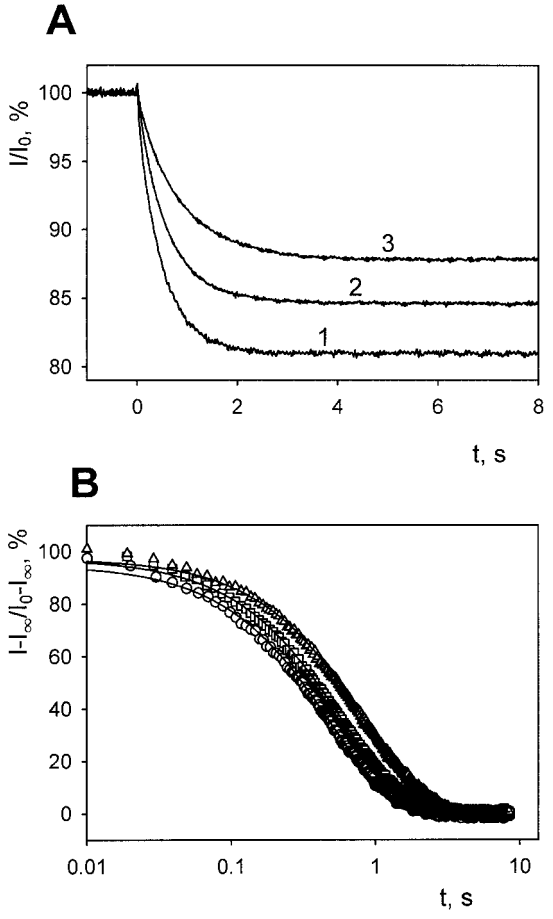

FIGURE 6 The kinetics of the decrease in the gramicidin-EDA-mediated current (*I*) through BLM in the presence of 1 μM $AlPcS_3$ after a flash of visible light (at zero time) in the control (*curve 1*), and in the presence of 0.06 μg/ml Konig's polyanion (*curve 2*), and 3 μg/ml Konig's polyanion (*curve 3*) at both sides of BLM. The experimental data points were plotted as (I/I_0) 100% in panel *A* and replotted as $(I - I_\infty)100\%/(I_0 - I_\infty)$ versus the time (logarithmic scale) in panel *B* where I_0 is the initial current and I_∞ is the stationary value of the current after a flash. Data were fitted with single exponentials with characteristic times $\tau = 0.49$ s (*curve 1*), $\tau = 0.58$ s (*curve 2*) and $\tau = 0.85$ s (*curve 3*). The initial value of the current (I_0) was $\sim$1 μA.

The effect of Konig's polyanion depended on the number of positive charges on the gramicidin molecules. In fact, the addition of 0.06 μg/ml Konig's polyanion at both sides of the membrane produced only small effects on τ for gram-EDA having a single positive charge at pH 7 (Fig. 6). Even at high polyanion concentrations (3 μg/ml, see of Fig. 6, *curve 3*), τ increased only twofold (from 0.49 to 0.85 s).

In Fig. 7 the characteristic time of gram-TAEA photoinactivation (*curve 1*) calculated from the single-exponential approximation of the kinetics is plotted versus the concentration of Konig's polyanion. It is seen that the dependence of τ on the concentration includes three regions. At low concentrations, the polyanion did not produce any effect on τ. Upon exceeding a certain (threshold) concentration, τ began to grow progressively and finally reached the maximum value. Further raising of the polyanion concentration led to a gradual decrease in τ. By contrast, only a slight increase in τ of gram-EDA was detected upon increasing the concentration of Konig's polyanion (Fig. 7, *curve 2*).

It is reasonable to expect that the effect of the polyanion on τ is dependent on the ionic strength of the bathing solution. Figure 8 shows the dependence of τ on the concentration of potassium chloride in the bathing solution at a constant concentration of the polyanion (note that, except for single-channel experiments and the data of Fig. 3, the results presented in all the other figures are obtained at 50

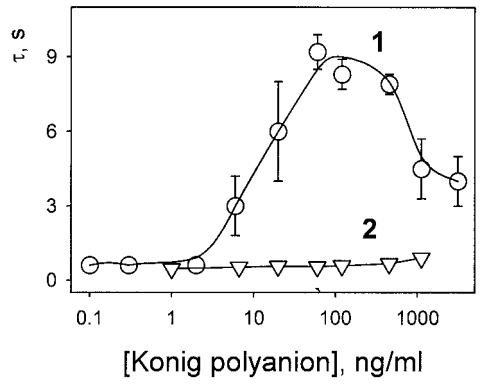

FIGURE 7 The dependences of the characteristic time (τ) of gram-TAEA (*curve 1*) and gram-EDA (*curve 2*) photoinactivation measured in the presence of Konig's polyanion on its concentration. The polyanion was added at both sides of the BLM. The initial value of the current for each measurement of τ was ~1 μA. The values of τ were obtained from single exponential approximation.

mM KCl). It is seen that the increase in the ionic strength led to the reduction of the polyanion effect on τ.

We tested different other polyanions in the system with gram-TAEA, namely double-stranded and single-stranded DNA, RNA from tobacco mosaic virus, polyglutamic acid, and polyacrylic acids of different molecular weights (5000 and 250,000). None of these increased the value of τ. It was found that modified polyacrylic acid, however, was effective, although at very high concentrations (Fig. 9). The decelerated kinetics of the current photoinactivation also had pronounced deviations from the monoexponential curve in the presence of high concentrations of modified polyacrylic acid as it was in the case of Konig's polyanion (Fig. 9 *A*, *curve 2*). The concentration dependence of the effect of modified polyacrylic acid is presented in Fig. 9 *B*.

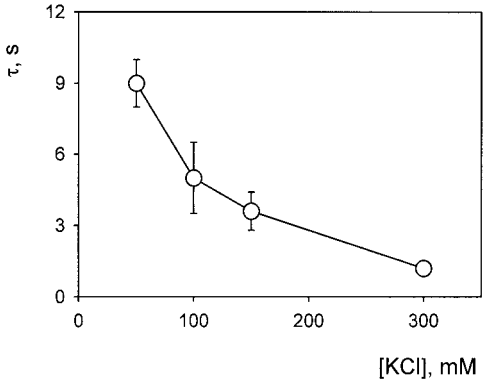

FIGURE 8 The dependence of the characteristic time of gram-TAEA photoinactivation (τ) measured in the presence of 0.06 μg/ml Konig's polyanion on the concentration of KCl in the bathing solution. Polyanion was added at both sides of the BLM. The initial value of the current for each measurement of τ was approximately 1 μA. The values of τ were obtained from single exponential approximation.

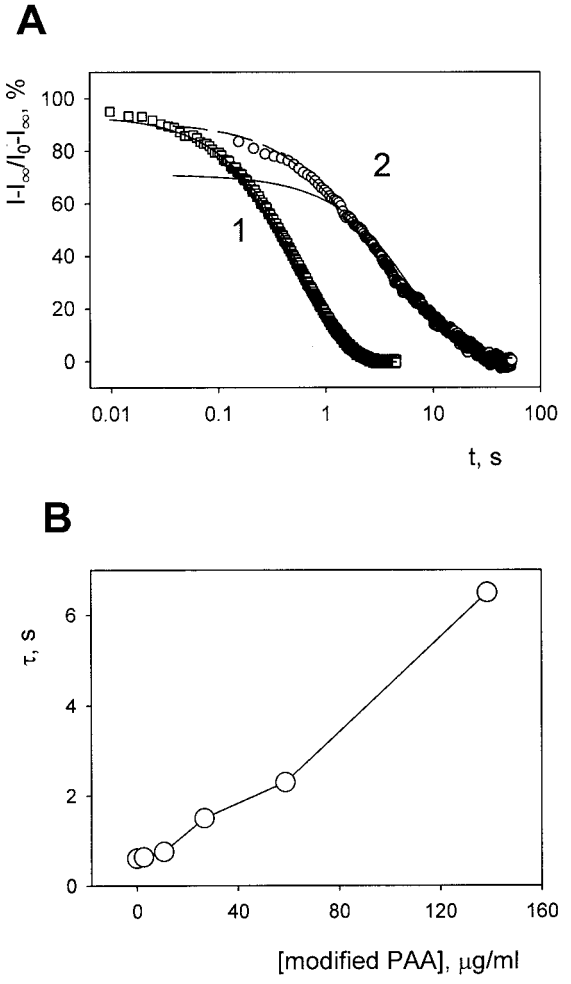

FIGURE 9 (*A*) The kinetics of the decrease in the gram-TAEA-mediated current (I) through BLM in the presence of 1 μM $AlPcS_3$ after a flash of visible light (at zero time) in the control (*curve 1*), and in the presence of 140 μg/ml modified polyacrylic acid (*curve 2*) at both sides of BLM. The experimental data points were fitted with single exponentials (*solid curves*) with characteristic times $\tau = 0.60$ s (*curve 1*), and $\tau = 6.5$ s (*curve 2*). In the case of curve *2*, double exponential fitting $I = I_0 + \alpha_1\exp(-t/\tau_1) + \alpha_2\exp(-t/\tau_2)$ (*dashed curve*) was also calculated with the following parameters: $\alpha_1 = 52\%$, $\tau_1 = 10.4$ s, $\alpha_2 = 48\%$, $\tau_2 = 1.5$ s. The data presented as $(I - I_\infty)100\%/(I_0 - I_\infty)$ versus the time (logarithmic scale), where I_0 is the initial current and I_∞ is the stationary value of the current after a flash. (*B*) The dependence of the characteristic time of gram-TAEA photoinactivation (τ) measured in the presence of modified polyacrylic acid on its concentration. The polyacrylic acid was added at both sides of the BLM. The initial value of the current for each measurement of τ was approximately 1 μA. The values of τ were obtained from single exponential approximation.

As mentioned above, the addition of DNA or polyacrylic acid did not increase the value of τ even at concentrations that were much higher than the effective concentrations of Konig's polyanion. However, it should be noted that the subsequent addition of Konig's polyanion after the addition of these polyanions did not lead to the change in the value of τ (data not shown). These data showed that, although

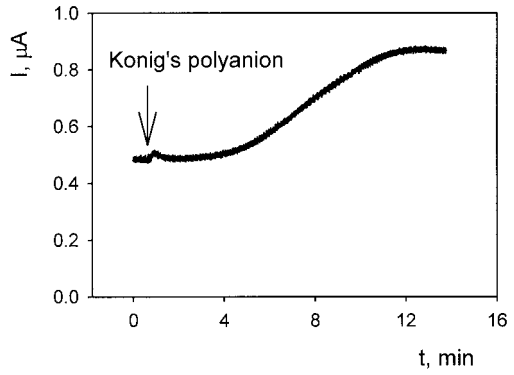

FIGURE 10 Effect of the addition of Konig's polyanion (5 ng/ml) at the moment marked by the arrow on the gram-TAEA-mediated current through BLM.

DNA did not change τ, it did interact with the membrane containing gram-TAEA.

It should be pointed out that the addition of Konig's polyanion to the bathing solutions (marked by the arrow in Fig. 10) led to an increase in the current through BLM mediated by gram-TAEA. It should be noted in connection with this that the photoinactivation kinetics (Figs. 2, 3, 6–9) were recorded 10–15 min after the addition of polyanions when a new steady-state level of the current was established. No change in the current was observed in the case of usual gramicidin, gA (data not shown).

DISCUSSION

As shown earlier (Rokitskaya et al., 1996, 1997), the characteristic time of gramicidin channel photoinactivation (τ) is determined by the time of establishment of the new equilibrium between transmembrane dimers and monomers of gramicidin after irreversible inactivation of a part of them. The dependence of τ on the BLM conductance induced by gram-TAEA (Fig. 3) enabled us to estimate the rate constants of channel formation and termination for this gramicidin analog. These values (see Results) are close to the kinetic constants of gA known from the literature ($K_R = 4.6 * 10^{13}$ mol^{-1}s^{-1}cm^2 and $K_D = 0.5$ s^{-1} for DPhPC at 26°C (Rokitskaya et al., 1996); or $K_R = 20 * 10^{13}$ mol^{-1}s^{-1}cm^2 and $K_D = 1.6$ s^{-1} for dioleoyllethicin at 25°C (Bamberg and Lauger, 1974); $K_R = 3 * 10^{13}$ mol^{-1}s^{-1}cm^2 and $K_D = 1.5$ s^{-1} for dioleoyllethicin at 21–23°C (Zingsheim and Neher, 1974), though the K_R value of gram-TAEA is noticeably lower than that of gA, which may be due to the increased polarity of the C-terminus of the gram-TAEA peptide hindering the process of formation of the transmembrane dimer (the channel state).

The polyanion/gram-TAEA system studied here can be considered as the inverted one to the polylysine/OPg system examined in our previous works (Krylov et al., 1998, 2000). Actually, it is the interaction between polyelectrolytes and the charged gramicidin with the signs of the charges switched. Several lines of evidence that will be discussed in detail below confirm the similarity of these two systems, namely: 1) the polyelectrolyte effects on the photoinactivation kinetics of charged gramicidins were pronounced only when the additions of the polyelectrolytes were made at both sides of the BLM (i.e., under the symmetric conditions), 2) both polyelectrolyte effects were prevented by increasing the ionic strength, and 3) both effects exhibited the bell-shaped dependence on the concentration of the polyelectrolytes.

It has been suggested previously that the increase in the characteristic time of photoinactivation observed upon binding of polylysine to OPg is due to formation of aggregates of OPg-polylysine clusters, i.e., τ is related to sequestering of OPg molecules into domains floating in the matrix of neutral phosphatidylcholine molecules (Krylov et al., 2000). The domain formation apparently results in reduction of lateral and rotational mobility of gramicidin molecules in a lipid bilayer, which manifests itself in the lengthening of the channel lifetime. The interaction of polyanions with positively charged gramicidin also can be described by this model. In fact, as seen from Fig. 5 *C*, channel openings of increased duration occurred very rarely after the addition of 100 ng/ml Konig's polyanion. However, under the conditions of measuring the integral current provided by simultaneous functioning of $\sim 10^6$ channels, the addition of Konig's polyanion at the same concentration resulted in the predominance of channels with a longer lifetime (Fig. 2). The dependence of the polyanion effect on the gram-TAEA concentration can be explained in terms of the above-mentioned model, in particular, by assuming aggregation of gram-TAEA channels induced by adsorption of polyelectrolyte chains on the membrane surface. Formation of large aggregates of gram-TAEA channels is expected to result in stabilization of the channels, i.e., in the lengthening of the channel lifetime, due to reduction of the peptide mobility in the aggregates. According to the data of ^{2}H-NMR spectroscopy (Mitrakos and Macdonald, 1998), binding of anionic polyelectrolytes causes deceleration of lateral diffusion and ensemble fluctuations of cationic amphiphiles in lipid bilayers. The requirement of adding Konig's polyanion at both sides of BLM to produce a marked effect on gram-TAEA photoinactivation kinetics indicates that molecules residing in both leaflets of the membrane are involved in the interaction with the polyanion, which results in domain formation.

The interaction of Konig's polyanion with lipid bilayers containing positively charged gramicidin is of electrostatic character, which is supported by the removal of the polyanion effect caused by polylysine, and by the dependence of the polyanion effect on the ionic strength of the bathing solution as evidenced by the data presented in Fig. 8. The interaction does not occur with neutral gA and is hardly

detected in the case of gram-EDA having a single positive charge (Fig. 6).

Mechanisms of segregation of lipid domains induced by interaction of polyelectrolytes with membranes containing charged lipids have been discussed in the literature (Denisov et al., 1998; May et al., 2000). It has been argued in (Hartmann et al., 1978; Sackmann et al., 1984; May et al., 2000) that binding of a multicharged basic protein to negatively charged lipid provokes a local change in line tension of BLM (i.e., elastic deformation of a lipid bilayer), which becomes the driving force of domain formation in membranes. Considering the effect of Konig's polyanion in terms of the latter model, it can be assumed that, following the step of electrostatic adsorption, Konig's polyanion can interact hydrophobically with neutral lipids of the membrane, thus altering its line tension. This two-step hypothesis allows us to explain the absence of the effect of other polyanions on gramicidin photoinactivation kinetics and the prevention of the effect of Konig's polyanion by the other polyanions. It was noted in the Results that a series of other polyanions studied here, except for modified polyacrylic acid, did not produce deceleration of gram-TAEA channel kinetics, though these polyanions prevented the effect of Konig's polyanion. Most likely, DNA and other polyanions actually bind to gram-TAEA on the membrane surface, which prevents the subsequent binding of Konig's polyanion, but does not cause the aggregation of gram-TAEA channels and thereby does not bring about an increase in τ. The reason might be that binding of DNA and other polyanions, except for modified polyacrylic acid, does not lead to changes in the line tension of lipid bilayers.

It can be supposed that the presence of abundant hydrophobic groups in polyelectrolyte molecules, such as Konig's polyanion and modified polyacrylic acid, provides their additional interaction with membrane lipids, leading to modulation of the line tension. This hypothesis is favored by the data of Tribet (1998) demonstrating that incorporation of hydrophobic groups (e.g., palmitoyl, cholesteroyl) provokes binding of charged polymers to bilayers formed of neutral lipids. Actually, it has been found by Maltseva et al. (2002) that Konig's polyanion, in contrast to unmodified polyacrylic acid, can adsorb on a neutral lipid membrane, thereby making it negatively charged. It is also relevant to this point that, according to a series of studies, myristoylation or palmitoylation of a number of basic proteins substantially increases their membrane affinity (Hancock et al., 1990; Sigal et al., 1994; Kim et al., 1994; Buser et al., 1994; McLaughlin and Aderem, 1995; Swierczynski and Blackshear, 1996; Murray et al., 1997, 1998; Bahr et al., 1998; Victor and Cafiso, 1998; Victor et al., 1999; Arbuzova et al., 2000).

As seen from Fig. 7, there was a maximum in the concentration dependence of the polyanion effect on the characteristic time of gram-TAEA photoinactivation. That is, further increasing the concentration of added Konig's polyanion led to partial reversal of the polyanion-induced increase in τ. The similar phenomenon was observed in our study of polylysine interaction with OPg channels (Krylov et al., 2000). This reversal most probably results from the electrostatic repulsion of polymer loops under the conditions of polymer competition for gram-TAEA charges, which leads to loosening of charged domains by including additional molecules of neutral lipid into them, i.e., to partial recovery of homogeneity in lateral distribution of two components (neutral and charged, see Macdonald et al., 1998). It is worth noting that disappearance of ordered domains in mixed bilayers formed of neutral and cationic lipids was observed earlier at high concentrations of added DNA (Hirsch-Lerner and Barenholz, 1998; Subramanian et al., 2000) and some other polyanions (Mitrakos and Macdonald, 1997).

The above consideration implies the existence of at least two populations of gram-TAEA channels in BLM, namely free channels and those arranged in domains. In fact, a single exponential did not suffice to describe the photoinactivation kinetics in the presence of Konig's polyanion. Under these conditions the kinetics was fitted well by a sum of two exponential components, with the characteristic time of one of them being close to that of the control. The exponential factor of the slower component supposedly corresponds to the channel lifetime in the domain phase.

In conclusion, it should be pointed out that certain advantages of our model system, including the simplicity and reliability of current measurements, make it useful to study mechanisms of protein-mediated domain formation in membranes. These mechanisms are of particular interest now in view of their involvement in processes of lipid raft formation and signal transduction (Brown and London, 2000; Simons and Toomre, 2000, and references in both).

APPENDIX

According to the analysis performed by Rokitskaya et al. (1996), the flash-induced decay of the gramicidin-mediated current, here called the kinetics of photoinactivation, represents the relaxation process after a concentration jump, i.e., the gramicidin monomer–dimer equilibration. The following formalism, based mainly on the consideration of Bamberg and Lauger (1973), describes the kinetics of the current transient after a flash of light.

The equilibrium between gramicidin monomers (A) and dimers (A_2) in the membrane depends on the reaction rate constants K_R and K_D as

$$A + A \underset{K_D}{\overset{K_R}{\leftrightharpoons}} A_2. \quad (1)$$

Let the flash occur at $t = 0$. The concentration of gramicidin in the membrane at $t = 0$ amounts to N. We assume also that the time of the exchange of gramicidin between the solution and the BLM exceeds considerably the time of relaxation, i.e., $N = \text{const}$ at $t > 0$. N_1 and N_2

correspond to the surface concentrations of A and A_2 in the BLM, respectively, so that

$$N_1 + 2N_2 = N = \text{const.} \quad (2)$$

As a result of the relaxation process, the surface concentrations of A and A_2 reach their equilibrium values, $N_{1\infty}$ and $N_{2\infty}$, respectively.

$$N = N_{1\infty} + 2N_{2\infty}, \quad (3)$$

$$\frac{N_{2\infty}}{(N_{1\infty})^2} = \frac{K_R}{K_D} = K, \quad (4)$$

where K is an equilibrium constant. According to Eq.1,

$$\frac{dN_2}{dt} = K_R(N_1)^2 - K_D N_2. \quad (5)$$

Assuming $y = N_2/N$ and taking into account Eq. 2, Eq.5 can be rewritten as

$$\frac{1}{K_D}\frac{dy}{dt} = 4NKy^2 - (1 + 4NK)y + NK. \quad (6)$$

Eq.6 has the solution,

$$y(t) = y_\infty = \frac{(y_\infty - y_0)qe^{-t/\tau}}{1 + q - e^{-t/\tau}}, \quad (7)$$

where

$$q = \frac{\sqrt{1 + 8NK}}{4NK(y_\infty - y_0)}, \quad (8)$$

$$1/\tau = K_D\sqrt{1 + 8NK} = K_D + 4K_R N_{1\infty}. \quad (9)$$

The BLM current (I) is proportional to the number of conducting dimers N_2 and therefore to y. Given small deviations from the equilibrium, at low energy of flashes,

$$\frac{I(t) - I_0}{I_\infty - I_0} = 1 - e^{-t/\tau}.$$

Therefore, this consideration suggests that the gramicidin-mediated current transient after a flash of light should follow monoexponential kinetics with the characteristic time (τ) determined by the rate constants of formation (K_R) and dissociation (K_D) of gramicidin dimers. The values of K_R and K_D can be calculated from the plot of $1/\tau$ versus $(\lambda_\infty)^{0.5}$, according to the following equation derived from the Eq. 9 (Rokitskaya et al., 1996):

$$\frac{1}{\tau} = K_D + 4\sqrt{\frac{K_R K_D \lambda_\infty}{N_A \Lambda}}, \quad (10)$$

where λ_∞ is the stationary BLM conductance after the light flash, N_A is Avogadro's number, and Λ is the single-channel conductance.

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